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FINAL REPORT
CYTOCHEMISTRY OF MALARIA
JUNE, 1979

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6 Cytochemistry of Malaria Under Treatment

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June 1979

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The Institute of Pathology, Case Western Reserve University
Cleveland, Ohio 44106

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Cytochemistry of malaria under treatment.

Masamichi Aikawa, M.D.

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Finally we examined host cell entry by malaria merozoites by electron microscopy. This study showed that the apical end of the merozoite forms a junction with the erythrocyte membrane. As this junction moves along the membrane, an invagination is created in the erythrocyte membrane. This indicates that the movement of the junction during invasion is an important component of the mechanisms by which the merozoite enters the erythrocyte.

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Summary

Several aspects relating to malaria were studied during these contract years. They include: 1) effects of antimalarial drugs on malarial parasites, 2) cytochemistry on the parasites, 3) host cell changes by malaria infection, 4) antibody-induced changes of malarial parasites, and 5) interaction between host cells and parasites.

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0 Foreward

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care", as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Science - National Research Council.

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Detailed Report

As stated in the original research contract application, the objectives of this research were to study the effects of selected antimalarial drugs such as 8-aminoquinolines and their metabolites, 4-aminoquinolines, antifolates and antibiotics on malarial parasites and host cells by electron microscopy, cytochemistry and autoradiography. This approach offered an overall understanding of the action of antimalarial drugs on the parasites as well as on the host cells. In addition, host cell-parasite interaction and morphology of certain malarial parasites were proposed to study in this contract.

Results and Significance

a) The effects of antimalarial drugs on malarial parasites.

(1) Effects of 8-aminoquinolines.

The effects of 8-aminoquinolines including primaquine, pentaquine, a metabolite of pentaquine and isopentaquine on the exoerythrocytic stages of P. fallax were investigated by electron microscopy since these drugs were known to be effective on the exoerythrocytic stages. This study revealed markedly swollen mitochondria of the parasite, indicating that 8-aminoquinolines have a primary effect on the parasite mitochondria. Electron microscope-autoradiography showed a concentration of tritiated primaquine in the mitochondria. Therefore, 8-aminoquinolines appear to primarily concentrate in the parasite mitochondria to interfere with the parasite's mitochondria. On the other hand, host cell mitochondrias appeared not to be affected by the drugs.

(2) Effects of 4-aminoquinolines.

Chloroquine, one of the 4-aminoquinolines is effective against the erythrocytic stages of most strains of malarial parasites. Electron microscopy revealed that the first morphologic changes after administration of chloroquine were in the parasite food vacuoles containing host cell cytoplasm. This indicates that chloroquine becomes highly concentrated in the erythrocytes infected by malarial parasites and enters into the parasite as it ingests red cell cytoplasm. Electron microscope autoradiography performed on the erythrocytic stages of a rodent malarial parasite, P. berghei, after exposure to tritiated chloroquine showed that ^3H -chloroquine became selectively localized within the parasite food vacuoles 1 hour after administration of the drug. This study, therefore, demonstrated that the food vacuoles containing red cell cytoplasm were the primary site of chloroquine concentration within the parasite.

(3) Effects of Antifolates

Pyrimethamine is another antimalarial drug which possesses schizonticidal activity in both tissue and blood phases. Although pyrimethamine is known to inhibit folic acid metabolism of malarial parasites by binding with folic acid reductase, the cytological effect on the parasite was not understood. When pyrimethamine was given to chickens infected with P. gallinaceum, the nuclear division of the parasite was arrested at metaphase and no further nuclear division processes took place. Also, a coordination between the process of nuclear division and cytokinesis was disrupted.

(4) Effects of Antibiotics.

The clinical and morphologic effects of antibiotics, clindamycin and

N-demethyl-4'-partyl clindamycin were evaluated using P. knowlesi in rhesus monkeys. Both compounds cured blood-induced infections when administered daily for five consecutive days. Morphologic changes within the parasite associated with drug action were observed in the parasite ribosomes 24 hours after drug administration. Affected ribosomes showed electron-lucent zones. During the following 24 hours these changes became more prominent with foci in which disintegrated ribosomes were replaced by fine fibrillar material. These studies indicate that clindamycin and its analog affect primarily the ribosomes and their mode of action is different from those of the above-mentioned antimalarials.

From these electron microscopic studies using different antimalarials it is apparent that different antimalarials act on different sites of the parasites. Each antimalarial has a specific concentration site and has specific mode of action. Based on these studies, antimalarials can be classified according to the site of drug concentration.

b) Electron microscope-cytochemistry.

(1) Localization of acid phosphatase activity within malarial parasites.

Acid phosphatase activity had been demonstrated in various protozoa by means of electron microscope-cytochemistry. However, there was a lack of bio- and cytochemical evaluation of this enzyme in malarial parasites. Therefore, we undertook an investigation to demonstrate the ultra-structural localization of acid phosphatase in the erythrocytic stages of P. gallinaceum and P. berghei. Acid phosphatase activity was demonstrated in the food vacuoles and endoplasmic reticulum of both parasites. This study confirmed the food vacuoles as the site of digestion of erythrocyte

cytoplasm. Acid phosphatase, one of the hydrolytic enzymes known to be associated with lysosomes assists in the digestive vacuoles. The demonstration of acid phosphatase activity, thus, confirmed the functional role of the food vacuoles of malarial parasites. Since chloroquine was demonstrated to localize within the food vacuoles, chloroquine may affect this enzyme to infer the digestive system of malarial parasites.

(2) Electron microscope-cytochemical method for differentiating membranes of host erythrocytes and malarial parasites.

In order to interpret the results of biochemical and immunological studies on "erythrocyte-free" - malarial parasites, one must consider the influence of host cell membranes that invariably contaminate such preparations, regardless of the isolation method utilized. Determining the percentage of parasites entrapped in erythrocyte membranes and quantitating host cell debris is, therefore, an essential procedure in such studies. Therefore, morphologic and cytochemical surface characteristics of isolated malarial parasites (*P. berghei*) and host erythrocytes were compared electron microscopically.

Erythrocytes had uniform, finely granular surfaces. Free-parasites exhibited a variety of sizes, shapes and surface textures. Results of cytochemical analyses using a sialophilic colloidal iron stain indicated that the surface of isolated malarial parasites lacked exposed sialic acid groups which give rise to a net negative surface charge common to most mammalian cells. Biochemical assay demonstrated that malaria parasites contained about half the amount of sialic acid per cent weight than control red cell

extracts. Similarly, external acidic mucopolysaccharide coats of free-parasites, as revealed by ruthenium red staining, are extremely thin as compared to the thick glycocalyx layer of red cells.

Lipoidal plaques at the surfaces of bounding plasma membranes were localized by lipophilic-iron colloid staining. The distribution of lipid plaques, revealed by bound colloid, at the surface of parasites and red cells were similar. However, surfaces of malarial parasites were intensely stained with patchwork patterns of bound lipophilic colloid more closely knit than those exhibited by erythrocytes. Such findings indicated differences in the size and numbers of exposed phospholipid areas at the surfaces of limiting membranes of host cells and parasites.

Because of the differences, limiting membranes of malarial parasites and host erythrocytes stained differently when exposed to positively charged iron colloid. Negatively charged red cell membranes bind colloid, whereas parasite membranes do not. This selectivity in colloidal iron uptake by the two types of membranes can be utilized as an aid in visualizing the amounts of contaminating host cell membranes in free-malarial parasites preparations and in related cell-free membrane extracts.

c) Host cell changes induced by malaria infection.

(1) Changes of erythrocytes infected by P. vivax and P. cynomolgi.

Host cells infected by certain species of malarial parasites below characteristic morphological alterations. The changes in the erythrocytes infected by vivax type malarial parasites are among the most striking. However, the nature of these changes was not known. Therefore, we

studied the changes in erythrocytes infected by P. vivax and P. cynomolgi by electron microscopy together with immuno-cytochemistry. The host cell changes include formation of caveola-vesicle complexes along the erythrocyte plasmalemma and formation of clefts and large vacuoles in the cytoplasm.

The caveola-vesicle complexes consist of caveola surrounded by several vesicles in alveolar fashion. They appear to correspond with Schuffner's dots observed by light microscopy, because of their pattern of distribution and their size. Also, horseradish peroxidase labelled antibody stains the complexes in a manner analogous to the way that Schuffner's dots are stained with fluorescein-labelled antibody against P. vivax. The caveola-vesicle complexes are pinocytotic in origin because they pick up ferritin particles.

(2) Changes of erythrocytes infected by P. simiovale and P. fieldi.

Schuffner's dots also appear in erythrocytes infected by ovale-type malarial parasites. Since vivax-type parasites induce an alteration of the plasmalemma of infected erythrocytes as stated in (1), we examined erythrocytes infected with P. simiovale and P. fieldi. Electron microscopy of erythrocytes infected by these parasites revealed caveola-vesicle complexes similar to those found in vivax-type parasites. This finding further confirmed our previous contention that the caveola-vesicle complexes are Schuffner's dots seen by light microscopy. In addition, the erythrocytes infected by P. simiovale and P. fieldi showed electron-dense excrescences which are located at the protruded tip of the erythrocytes. These changes may be the basis for the characteristic fimbriation of

ovale-type infected erythrocytes on Giemsa stained blood films.

d) Antibody-induced ultrastructural changes of malarial parasites.

(1) Merozoite changes.

Immunity has been induced against the sporozoites and erythrocytic stages of infection. In the case of immunity against erythrocytic infection, transfer of serum or IgG from a host immune to malaria suppressed or greatly reduced parasitemia in a non-immune host.

In this study, the effect of immune serum on merozoites of P. knowlesi was evaluated by: 1) direct observation of merozoites after release from erythrocytes, 2) estimation of merozoites of agglutination, 3) quantification of invasion rate, and 4) observation on the ultrastructure of merozoites in immune serum.

Immune serum agglutinated merozoites of P. knowlesi in culture. They attached to erythrocytes, but were usually unable to invade. Marked aggregation of merozoites was associated with reduced invasion of erythrocytes in these cultures. The agglutination and reduced invasion were immunologically specific, for the effect of serum was greatest against homologous strains of P. knowlesi. Merozoite agglutination was caused by the binding of surface coats on adjacent parasites. This coat appeared on the plasma membrane of merozoites after it was exposed to culture medium, both with and without immune serum. The coat consisted of protein or glycoprotein, since it was susceptible to trypsin treatment. It appears that antibodies directed against this surface coat are crucial for the reduced invasion of the erythrocytes.

(2) Sporozoite changes.

Immunization with irradiated sporozoites produces a considerable degree of protection against rodent, simian and human malaria. This protection is in part antibody-mediated. Antibodies neutralize sporozoites, i.e., abolish their infectivity, and cause the formation of a thread-like precipitate on the parasites. A study was undertaken to characterize the ultrastructural aspects of antibody-sporozoite interaction. Gradient concentrated sporozoites of P. berghei and P. cynomolgi were incubated with immune and normal sera. Electron microscopy revealed the presence of a prominent, thick coat surrounding the outer membrane of the sporozoites incubated in immune serum. The inner structure of these parasites appeared to be relatively unaltered. The coat was absent or minimal on parasites incubated in normal serum. Incubation of immune serum pre-treated sporozoites with rabbit anti-mouse gamma globulin conjugated with hemocyanin demonstrated the participation of immunoglobulin in the formation of this surface deposition. Coat formation occurred also on the surface of metabolically inactive, non-secreting parasites such as formalin treated sporozoites and parasites kept on ice. Scanning electron microscopy and negative staining indicated that the anterior end of the parasites was free of coat deposition and that the thread-like precipitate was located at the posterior end of the sporozoites.

e) Interaction between host cells and malarial parasites.

(1) Host cell entry by P. knowlesi merozoites.

Invasion of erythrocytes by merozoites of P. knowlesi was investigated by electron microscopy. The apical end of the merozoite makes initial

contact with the erythrocyte, creating a small depression in the erythrocyte membrane (Fig. 1). The area of the erythrocyte membrane to which the merozoite is attached becomes thickened and forms a junction with the plasma membrane of the merozoite. As the merozoite enters the invagination in the erythrocyte surface, the junction which is in the form of a circumferential zone of attachment between the erythrocyte and merozoite, moves along the confronted membranes to maintain its position at the orifice of the invagination (Figs. 2 & 3). When entry is completed, the orifice closes behind the parasite in the fashion of an iris diaphragm and the junction becomes a part of the parasitophorous vacuole (Fig. 4). The movement of the junction during invasion is an important component of the mechanism by which the merozoite enters the erythrocyte.

(2) Effect of cytochalasin B on host cell entry by merozoites.

After our understanding of the interaction between the merozoite and the normal erythrocyte, our attention was focused on the structural and functional differences between the normal invasion sequence and the defective interaction between Duffy negative human erythrocytes and malarial merozoites. Since identification of the defect in invasion of Duffy negative erythrocytes could not be explored by previously available techniques, we developed a method for the isolation of the attachment phase by using cytochalasin B. Cytochalasin B is known to affect microfilaments and glucose transport of cells.

When cytochalasin B-treated merozoites of P. knowlesi were incubated with rhesus erythrocytes. Duffy positive human erythrocytes and Duffy negative human erythrocytes, light microscopy showed that the apical

end of the merozoites were oriented toward these erythrocytes. However, the invasion processes did not advance further. Electron microscopy showed the attachment between the apical end of a treated merozoite and rhesus erythrocyte, forming a junction similar to that seen for an untreated merozoite (Fig. 5), but no further steps in the invasion process took place. The attachment between a cytochalasin treated merozoite and Duffy positive human erythrocyte is identical to that seen for rhesus erythrocyte (Fig. 6). In contrast, no junction formation was seen between a Duffy negative human erythrocyte and treated merozoites. The apical end of the merozoite is oriented toward the erythrocyte, but instead of a junction, the erythrocyte is about 120 mu away from the merozoite and they were connected by thin filaments (Fig. 7). Therefore, we concluded that: 1) cytochalasin B-treated merozoites attach to host erythrocytes and forms a junction, but cytochalasin blocks the movement of this junction preventing further invasion, and 2) the absence of junction formation with Duffy negative human erythrocytes may indicate that the Duffy associated antigen acts as a receptor for junction formation or a determinant on Duffy negative erythrocytes blocks the junction formation.

Figure Legends

- Fig. 1 Electron micrographs of a merozoite contacting an erythrocyte. X 60,000.
- Fig. 2 Erythrocyte entry by a merozoite. The invagination of the erythrocyte is created by a merozoite. X 54,000.
- Fig. 3 A further advanced stage of erythrocyte entry by a merozoite. The junction (arrow) is formed between the thickened membrane of the erythrocyte and merozoite plasmalemma. X 50,000.
- Fig. 4 A merozoite is inside of our erythrocyte. However, the posterior end is still attached to the erythrocyte membrane. X 50,000.
- Fig. 5 An attachment between a rhesus erythrocyte and cytochalasin B-treated merozoite (arrow). X 60,000.
- Fig. 6 An attachment between a Duffy positive human erythrocyte (E) and a cytochalasin B-treated merozoite (M). X 60,000.
- Fig. 7 Interaction between a Duffy negative human erythrocyte and a cytochalasin B-treated merozoite. Note filaments between the merozoite and erythrocyte. X 55,000.

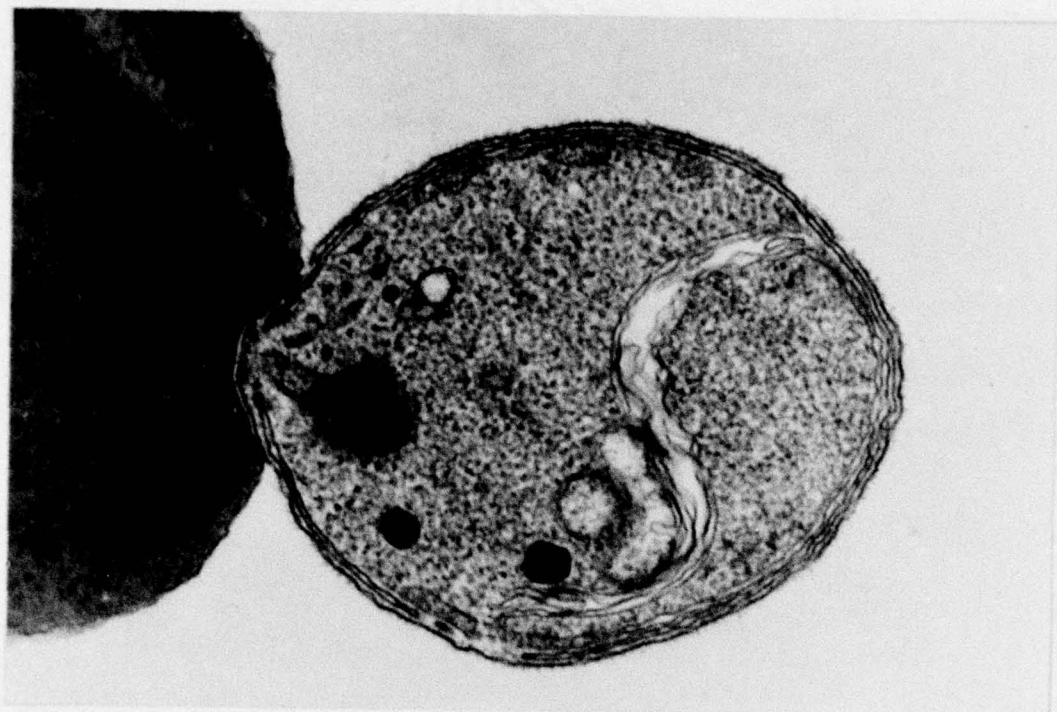


Fig 1

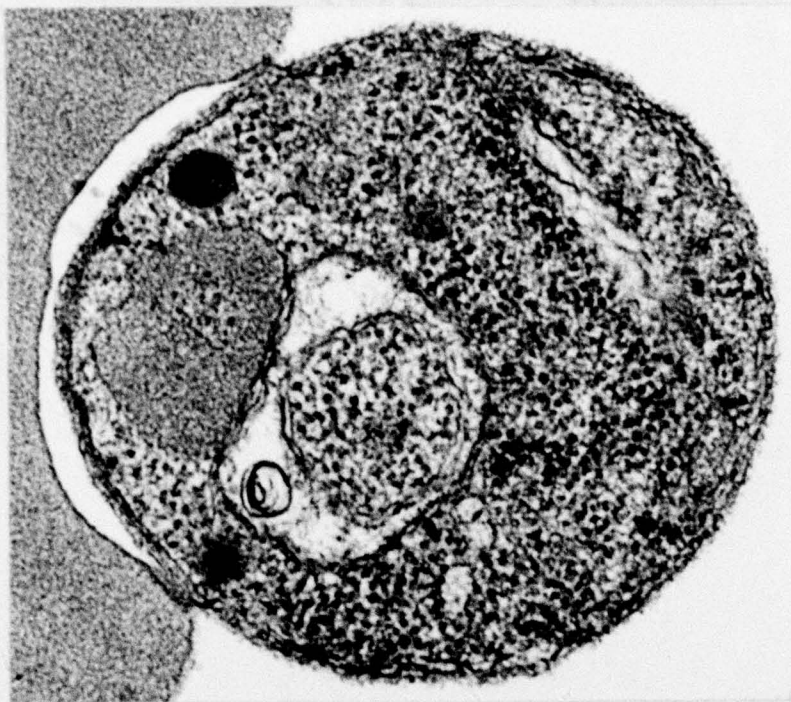


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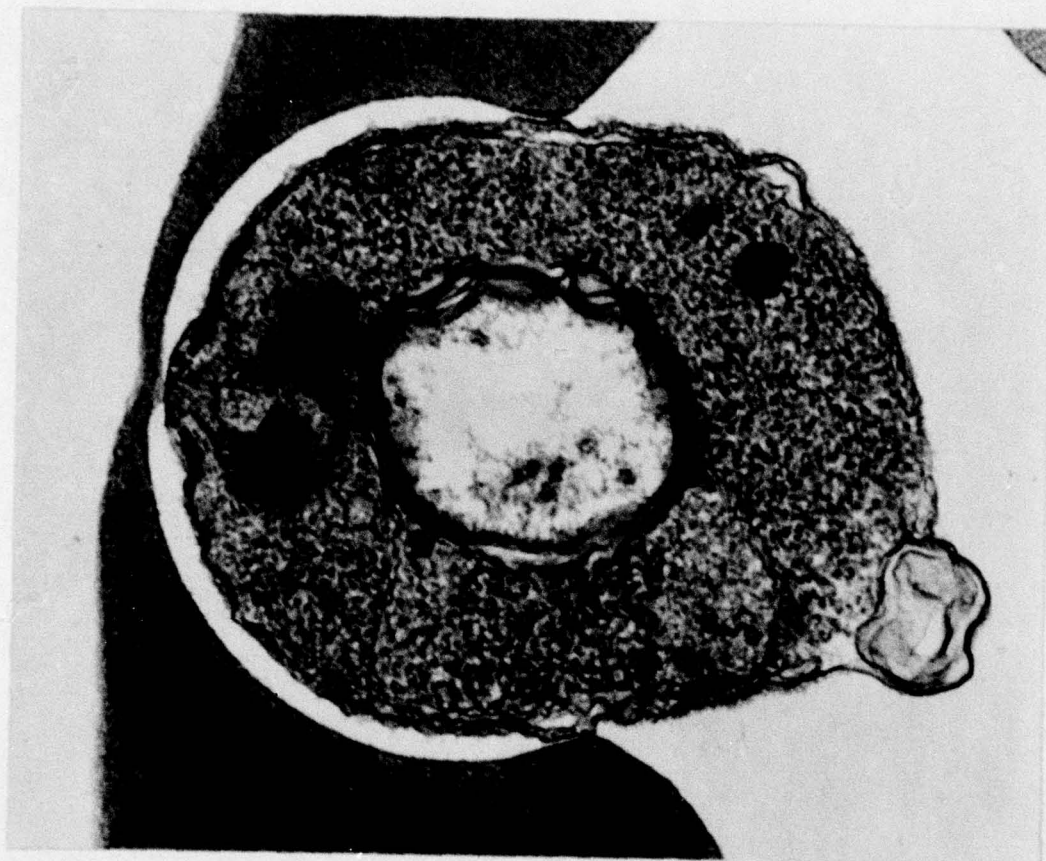


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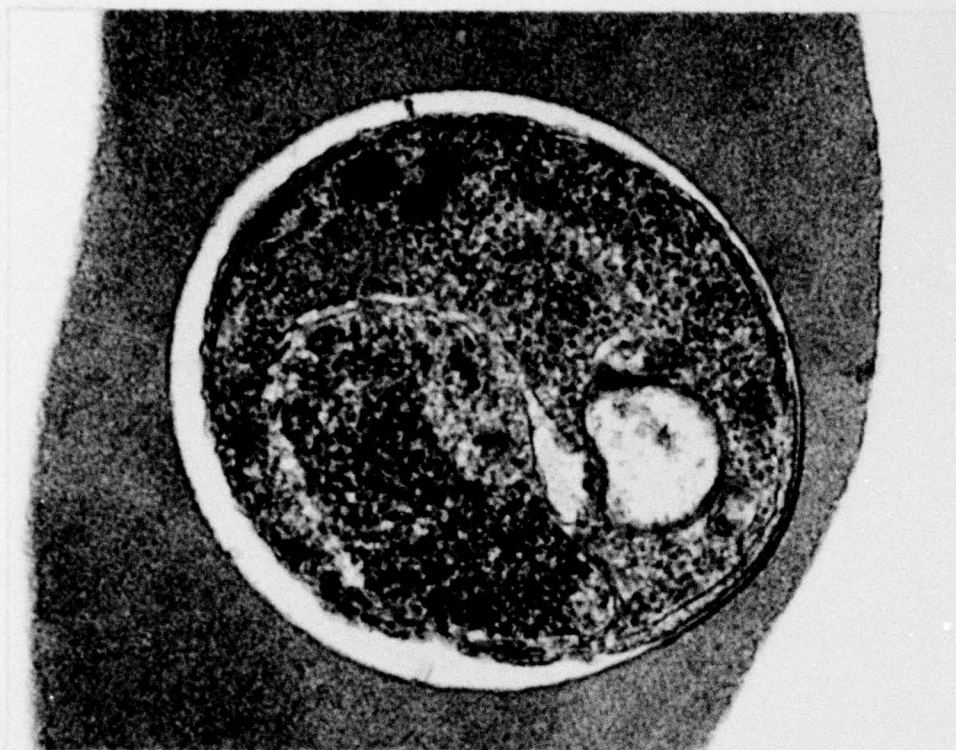


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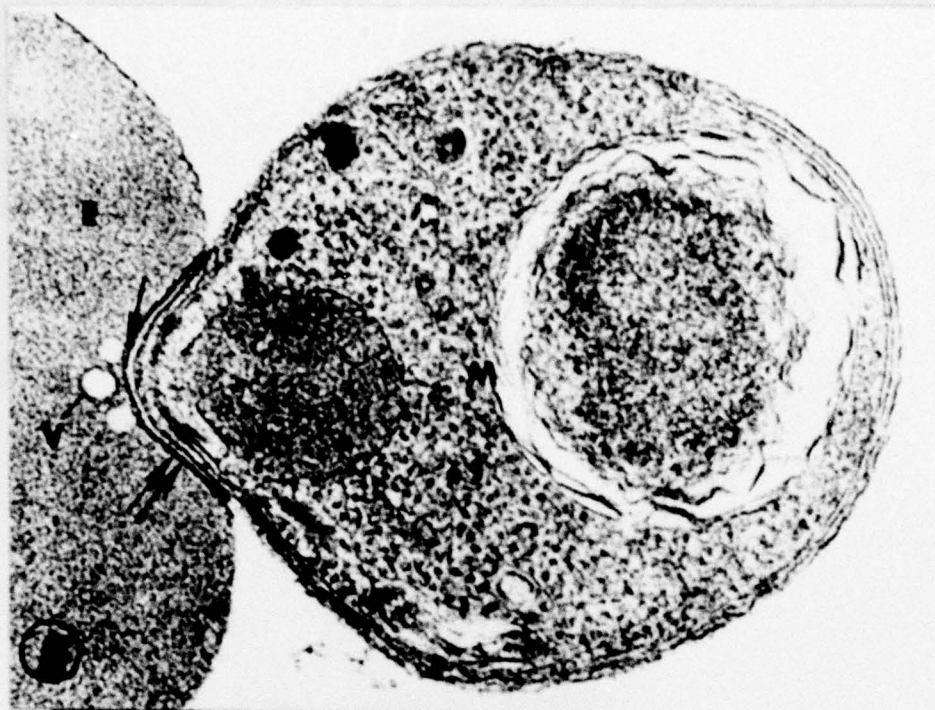


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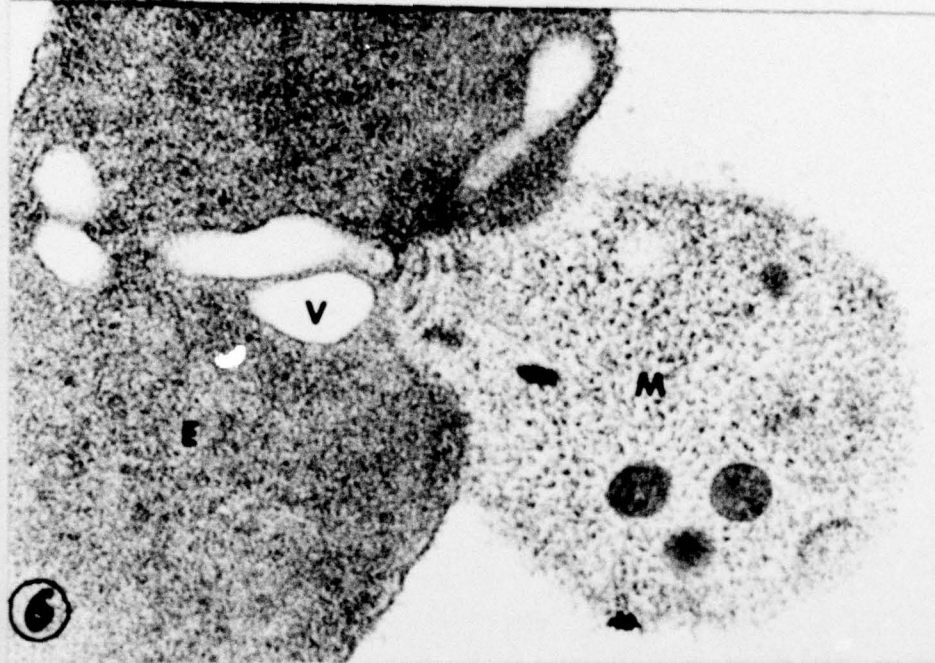


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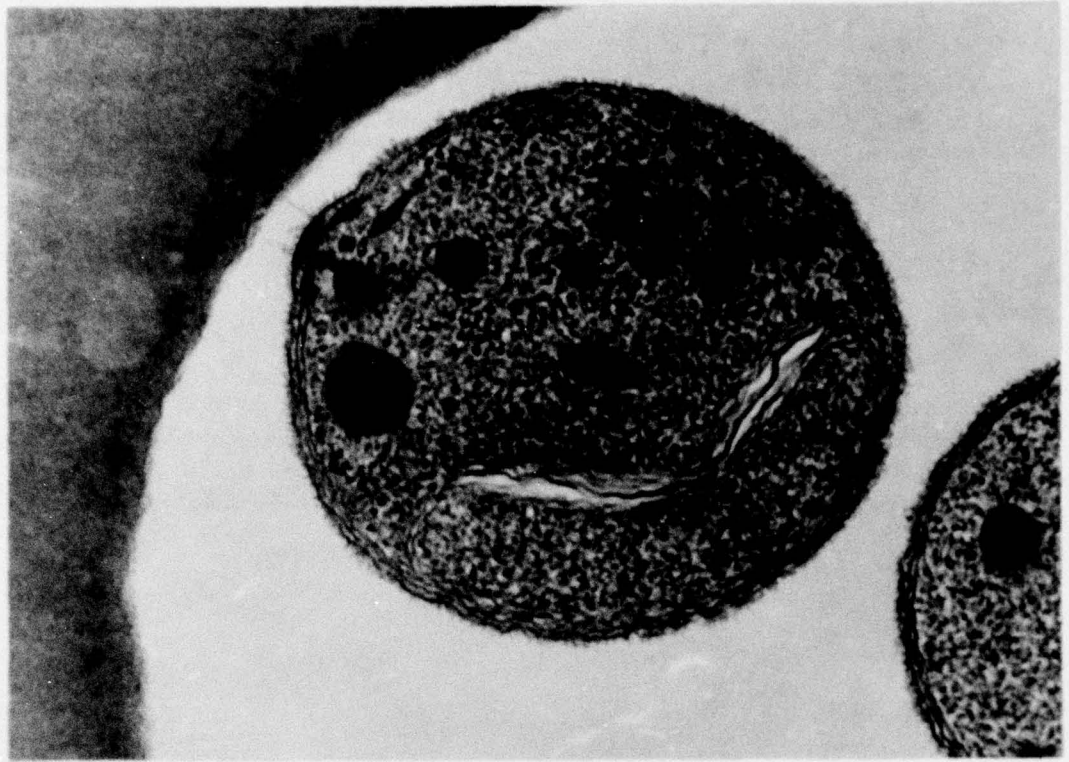


Fig 7

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